RayBio® Human Angiogenesis Antibody Array C Series 1000

Patent Pending Technology

User Manual (Revised February 4, 2009)

RayBio® Human Angiogenesis Antibody Array C series 1000
Combination of Arrays 1 & 2 (Cat# AAH-ANG-1000)

RayBio® Human Angiogenesis Antibody Array 1 (Cat# AAH-ANG-1)
RayBio® Human Angiogenesis Antibody Array 2 (Cat# AAH-ANG-2)

Please read manual carefully before starting experiment

RayBiotech, Inc.

We Provide You with Excellent
Protein Array Systems and Service

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RayBiotech, Inc.

RayBio® Human Angiogenesis Antibody Array C Series 1000 Protocol

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Angiogenesis antibody arrays are RayBiotech patent-pending technology.

RayBio® is the trademark of RayBiotech, Inc.

I. Introduction

All cell functions, including cell proliferation, cell death and differentiation, as well as maintenance of health status and development of disease, are controlled by a multitude of genes and signaling pathways. New techniques such as cDNA microarrays have enabled us to analyze global gene expression [1-3]. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows a disparity between the relative expression levels of mRNA and their corresponding proteins [4]. Therefore, analysis of the protein profile is critical. Currently, two-dimensional polyacrylamide SDS page coupled with mass spectrometry is the mainstream approach to analyzing multiple protein expression levels [5,6]. However, the requirement of sophisticated devices and the lack of quantitative measurements greatly limit their broad application. Thus, effective study of multiple protein expression levels has been complicated, costly are time-consuming until now.

Our RayBio® Human Angiogenesis antibody array is the first commercially available protein array system [7-11]. By using the RayBiotech system, scientists can rapidly and accurately identify the expression profiles of multiple cytokines in several hours inexpensively.

The RayBiotech kit provides a simple array format, and highly sensitive approach to simultaneously detect multiple cytokine expression levels from conditioned media, patient's sera, cell lysate, tissue lysates and other sources.

Traditionally, cytokines are detected by using ELISA. However, RayBiotech's approach has several advantages over ELISA. First, and most importantly, our approach can detect many cytokines simultaneously. Secondly, sensitivity is greatly increased. As little as 4 pg/ml of MCP-1 can be detected using the protein array format. In contrast, at least 40 pg/ml of MCP-1 is required to produce a clear signal in an ELISA assay. Furthermore, the detection range is much greater than ELISA. For example, the detection range of IL-2 varies from 25 to 250,000 pg/ml using RayBiotech technology, whereas the detection range varies only within 100-1000 fold in a typical

ELISA. Therefore, the detection range is greater with protein array compared with ELISA. The variation is lower than ELISA as well. As determined by densitometry, the variation between two spots ranged from 0 to 10% in duplicated experiments. In contrast, variation (about 20%) in ELISA is much higher. Finally, the system is much quicker and can be much easier to adapt to high-throughput technique.

Pathway-specific array systems allow investigators to focus on the specific problem and are becoming an increasingly powerful tool in cDNA microarray systems. RayBiotech's first protein array system, known as RayBio® Human Angiogenesis antibody array, is particularly useful in comparison with the human cytokine cDNA microarray system. Besides the ability to detect protein expression, RayBiotech's system is a more accurate reflection of active cytokine levels because it only detects secreted cytokines, and no amplification step is needed. Furthermore, it is much simpler, faster, environmentally friendlier, and more sensitive.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cytokines. Cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation [12]. Cytokines are involved in most disease processes, including cancer and cardiac diseases. The interaction between cytokines and the cellular immune system is a dynamic process. The interactions of positive and negative stimuli, and positive as well as negative regulatory loops are complex and often involve multiple cytokines.

Without doubt, simultaneous detection of multiple cytokines provides a powerful tool to study cytokines.

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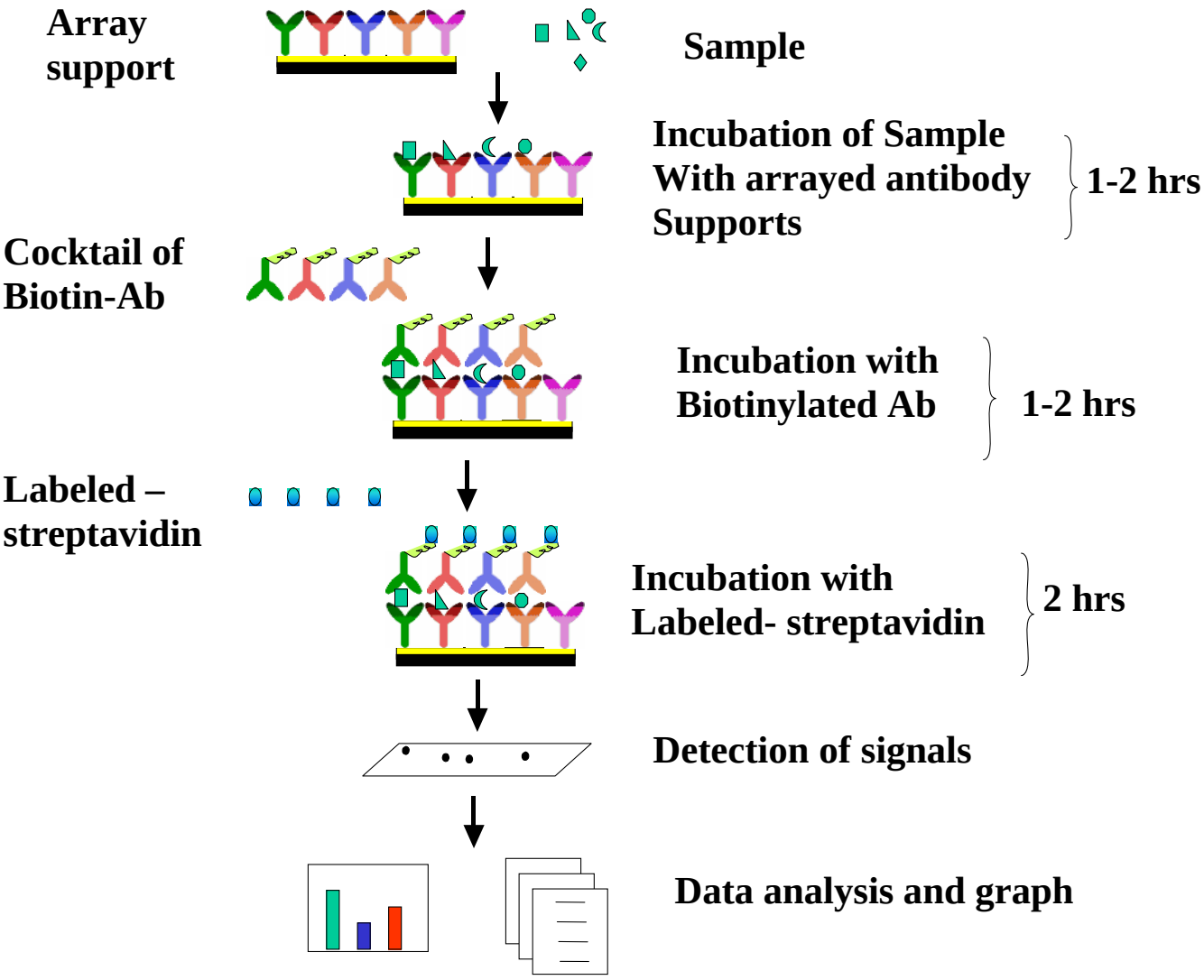
Here's how it works



II. Materials Provided

Upon receipt, all components of the RayBio® Human Cytokine Antibody Array kit should be stored at -20°C to -80°C. At -20°C to -80°C the kit will retain complete activity for up to 6 months. Once thawed, the array membranes and 1X Blocking Buffer should be kept at –20°C and all other component should be stored at 4°C. After thawing the reagents, the kit must be used within three months, and please use the kit within six months of purchase.

- RayBio® Human Angiogenesis antibody array membranes (2/4/8 array membranes 1 and 2/4/8 array membranes 2)
- Biotin-Conjugated Anti-Cytokines (1/2/4 tubes, each tube for two membranes)
- 1,000X HRP-Conjugated Streptavidin (50 µl)
- 1X Blocking Buffer (25/50 ml)
- 20X Wash Buffer I (10/20 ml)
- 20X Wash Buffer II (10/20 ml)
- 2X Cell Lysis Buffer (10/20 ml)
- Detection Buffer C (1.5/2.5 ml)
- Detection Buffer D (1.5/2.5 ml)
- Eight-Well Tray (1 each)
- Manual

Additional Materials Required

- Small plastic boxes or containers
- Orbital shaker
- Plastic sheet protector or SaranWrap
- Kodak X-Omat AR film (REF 165 1454) and film processor or Chemiluminescence imaging system

III. Overview and General Considerations

A. Preparation of Samples

- Use serum-free conditioned media if possible.
- If serum-containing media is required, use an uncultured media aliquot as a negative control sample, since many types of sera contain cytokines.
- For cell lysates and tissue lysates, we recommend using RayBio® Cell Lysis Buffer to extract proteins from cell or tissue (e.g. using homogenizer). Dilute 2X RayBio® Cell Lysis Buffer with H_2O (we recommend adding proteinase inhibitors to Cell Lysis Buffer before use). After extraction, spin the sample down and save the supernatant for your experiment. Determine protein concentration.
- We recommend using per membrane:
    - 1 ml of Conditioned media (undiluted), or
    - 1 ml of 2-fold to 5-fold diluted sera or plasma, or
    - 50-500 µg of total protein for cell lysates and tissue lysates (use ~200-250 µg of total protein for first experiment) ***Dilute the lysate at least 10 fold with 1 X blocking buffer.***

Note: The amount of sample used depends on the abundance of adipokines. More of the sample can be used if the signals are too weak. If the signals are too strong, the sample can be diluted further.

If you experience high background, you may further dilute your sample.

B. Handling Array Membranes

- Always use forceps to handle membranes, and grip the membranes by the edges only.
- Never allow the array membranes to dry during experiments.

C. Incubation

- Completely cover the membranes with sample or buffer during incubation, and cover the eight-well tray with lid to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Several incubation steps such as step 2 (blocking), step 3 (sample incubation), step 8 (biotin-Ab incubation) and step 11 (HRP-streptavidin incubation) may be done at 4°C for overnight, but make sure to cover the 8 well plate tightly to prevent evaporation.

IV. Protocol

A. Blocking and Incubation

1. Place one array membrane **1** (top left corner marked with "**1**"), and one array membrane **2** (top left corner marked with "**2**") into the same well of the provided eight-well tray ("1" or "2" marked side is the antibody printed side).

2. Add 2 ml 1X Blocking Buffer and incubate at room temperature for 30 min to block membranes. Make sure there are no bubbles between the membranes.

3. Decant Blocking Buffer from each container, and incubate membranes with sample at room temperature for 1 to 2 hours. Dilute sample using 1X Blocking Buffer if necessary.

Note: We recommend using 1.2 ml of undiluted conditioned media or 1.2 ml of 2-fold to 10-fold diluted sera or plasma or ~200-250 ug (range: 50-500 ug) of total protein for cell lysates and tissue lysates. ***Dilute the lysate at least 10 fold with 1 X blocking buffer. Add some samples between array membranes I and II. Make sure there are no bubbles between membranes.***

Note: The amount of sample used depends on the abundance of cytokines. More of the sample can be used if the signals are too weak. If the signals are too strong, the sample can be diluted further.

Note: Incubation may be done at 4°C for overnight.

4. Decant the samples from each container, and wash 3 times with 2 ml of 1X Wash Buffer I at room temperature with shaking. Please allow 5 min per wash. Dilute 20X Wash Buffer I with H_2O.

5. Wash 2 times with 2 ml of 1X Wash Buffer II at room temperature with shaking. Allow 5 min per wash. Dilute 20X Wash Buffer II with H_2O.

6. **From this step, place array membrane 1 marked with "1") into one well, array membrane 2 marked with "2") into another well.**

7. Prepare working solution for biotin-conjugated antibodies.

   Add 100 μl of 1x blocking buffer to the Biotin-Conjugated Antibody 1 tube. Mix gently and transfer all mixture to a tube containing 2 ml of 1x blocking buffer.

   Add 100 μl of 1x blocking buffer to the Biotin-Conjugated Antibody 2 tube. Mix gently and transfer all mixture to a tube containing 2 ml of 1x blocking buffer.

Note: the diluted biotin-conjugated antibodies can be stored at 4°C for 2-3 days.

8. Add 1 ml of diluted biotin-conjugated antibodies to each membrane (1 ml of diluted biotin-conjugated antibodies 1 to array membrane 1 marked with "1" and 1 ml of diluted biotin-conjugated antibodies 2 to array membrane 2 marked with "2"). Incubate at room temperature for 1-2 hours.

Note: incubation may be done at 4°C for overnight.

9. Wash as directed in steps 4 and 5.

10. Add 2 ml of **1,000** fold diluted HRP-conjugated streptavidin (e.g. add **2** µl of HRP-conjugated streptavidin to **1998** µl 1X Blocking Buffer) to each membrane.

*Note**: mix tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.***

11. Incubate at room temperature for 2 hours.

Note: incubation may be done at 4°C for overnight.

12. Wash as directed in steps 4 and 5.

B. Detection

*** Do not let the membrane dry out during detection. The detection process must be completed within 40 minutes without stopping.**

1. Proceed with the detection reaction.

Add 250 µl of 1X Detection Buffer ***C*** and 250 µl of 1X Detection Buffer ***D*** for one membrane; mix both solutions; Drain off excess wash buffer by holding the membrane vertically with forceps. Place membrane protein side up ("1"or "2" mark is on the protein side top left corner) on a clean plastic sheet (provided in the kit). Transfer the mixed Detection Buffer onto the membrane and incubated at room temperature for ***2*** minutes. Ensure that the detection mixture is completely and evenly covers the membrane without any air bubbles.

2. Drain off any excess detection reagent by holding the membrane vertically with forceps and touching the edge against a tissue. Gently place the membrane, protein side up, on a piece of plastic sheet ("1" or "2" mark is on the protein side top left corner). Cover with another piece

of plastic sheet on the array. Gently smooth out any air bubbles. Avoid using pressure on the membrane.

3. Expose the array to x-ray film (we recommend to use Kodak X-Omat AR film) and detect the signal using film developer. Or the signal can be detected directly from the membrane using a chemiluminescence imaging system.

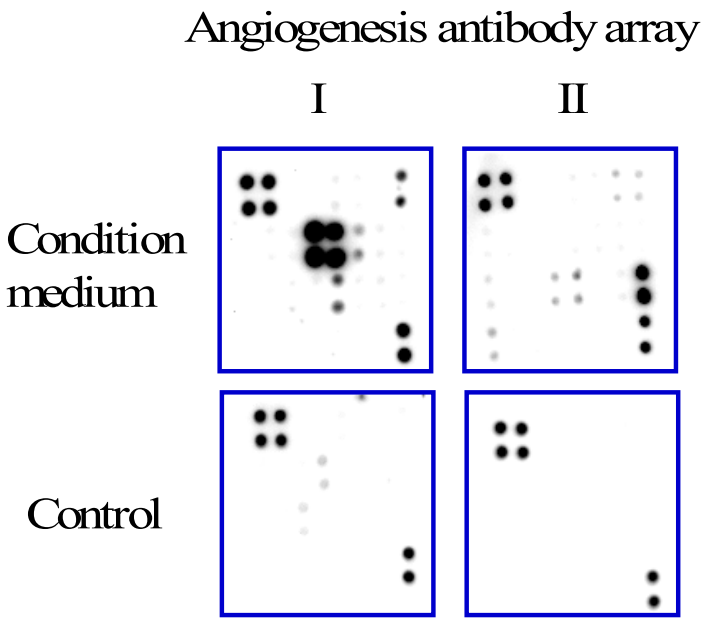
Expose the membranes for 40 seconds. Then re-expose the film according to the intensity of signals. If the signals are too strong (background too high), reduce exposure time (e.g. 5-30 seconds). If the signals are too weak, increase exposure time (e.g. 5-20 min or overnight), or re-incubate membranes overnight with 1x HRP-conjugated streptavidin, and redo detection on the second day.

4. Save membranes in –20 °C to –80 °C for future reference.

V. Interpretation of Results:

The following figure shows RayBio® Human Angiogenesis antibody array membranes C series 1000 probed with different patient's plasma. Membranes were exposed to Kodak X-Omat film at room temperature for 1 minute. The biotin-conjugated IgG produces positive signals, which can be used to identify the orientation and to compare the relative expression levels among the different membranes.

One important parameter is background. To obtain the best results, we suggest that several exposures be attempted. We also strongly recommend using a negative control in which the sample is replaced with an appropriate mock buffer according to the array protocol, particularly during your first experiment.



By comparing the signal intensities, relative expression levels of cytokines can be made. The intensities of signals can be quantified by densitometry. The positive control can be used to normalize the results from the different membranes being compared. The signals also can be detected and quantified by using a chemiluminescence-imaging device.

The **RayBio® Analysis Tool** is a program specifically designed for analysis of RayBio® Angiogenesis antibody arrays. This tool will not only assist in compiling and organizing your data, but also reduces your calculations to a "copy and paste." Call RayBiotech, Inc. at 770-729-2992 for ordering information.

RayBio® Human Angiogenesis Antibody Array 1

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
2	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
3	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
4	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
5	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
6	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Neg	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Neg	POS

Note: GRO detects CXCL1, CXCL2, CXCL3
Note: TGF-β1 detects only active form
Note: VEGF detects VEGF-165 and VEGF-121

RayBio® Human Angiogenesis Antibody Array 2

	a	b	c	d	e	f	g	h
1	POS	POS	NEG	NEG	Angiopoietin-1	Angiopoietin-2	Angiostatin	Endostatin
2	POS	POS	NEG	NEG	Angiopoietin-1	Angiopoietin-2	Angiostatin	Endostatin
3	G-CSF	GM-CSF	I-309	IL-10	IL-1α	IL-1β	IL-2	IL-4
4	G-CSF	GM-CSF	I-309	IL-10	IL-1α	IL-1β	IL-2	IL-4
5	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	Tie-2	TNF-α
6	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	Tie-2	TNF-α
7	u PAR	VEGF R2	VEGF R3	BLANK	BLANK	BLANK	BLANK	POS
8	u PAR	VEGF R2	VEGF R3	BLANK	BLANK	BLANK	BLANK	POS

We also offer Custom Human Cytokine Antibody Arrays. You can select the cytokines of interest from the following list and we will produce the customized array at an affordable price. For more information, please visit our website, **www.raybiotech.com**.

Human Custom Antibody Array List (285 proteins)

4-1BB/TNFRSF9	CNTF	GDNF	IL-18 R alpha	MIP-1 alpha	SCF
ACE-2	Cripto-1	GITR	IL-18 R beta	MIP-1 beta	SCF R
Activin A	CRP	GITR Ligand	IL-1ra	MIP-1 delta	SDF-1 alpha
Adiponectin/Acrp30	CTACK/CCL27	GM-CSF	IL-2	MIP-3 alpha	SDF-1 beta
Adipsin/Factor D	CTLA-4	GRO	IL-2 R alpha	MIP-3 beta	sgp130
AFP	CXCL16	GRO-a	IL-2 R beta	MMP-1	Shh N
AgRP(ART)	DAN	Growth Hormom	IL-2 R gamma	MMP-2	Siglec-5
ALCAM	Decorin	HB-EGF	IL-21 R	MMP-3	Siglec-9
Angiogenin	DKK-1	HCC-4/CCL16	IL-22	MMP-7	sTNF RII
Angiopoietin-1	DKK-3	hCGa, intact	IL-28A/IFN-lambda	MMP-8	sTNT RI
Angiopoietin-2	DKK-4	HGF	IL29/IFN-lambda 1	MMP-9	TACE
Angiostatin	DPPIV/CD26	HVEM	IL-3	MMP-10	TARC
ANGPTL4	DR6	I-309	IL-31	MMP-13	TECK/CCL25
AR (amphiregulin)	Dtk	ICAM-1	IL-4	MPIF-1	TGF-alpha
Axl	E-Cadherin	ICAM-2	IL-5	MSP a Chain	TGF-beta 1
B7-1(CD80)	EDA-A2	ICAM-3	IL-5 R alpha	NAP-2	TGF-beta 2
Bate2 M	EGF	IFN-gamma	IL-6	NCAM-1	TGF-beta 3
BCAM	EGF R	IGFBP-1	IL-6 sR	NGF R	Thyroglobulin
BCMA/TNFRSF17	EG-VEGF/PK1	IGFBP-2	IL-7	Nidogen-1/Entactin	Tie-1
BDNF	ENA-78	IGFBP-3	IL-8	NrCAM	Tie-2
beta IG-H3	Endoglin	IGFBP-4	IL-9	NRG1-beta 1/HRG1-beta 1	TIM-1
Betacellulin (BTC)	Endostatin	IGFBP-5	IL-9 R	NT-3	TIMP-1
bFGF	Eotaxin	IGFBP-6	Insulin	NT-4	TIMP-2
BLC	Eotaxin-2	IGF-I	IP-10	Oncostatin M	TIMP-4
BMP-4	Eotaxin-3	IGF-I sR	I-TAC/CXCL11	Osteopontin	TNF-alpha
BMP-5	EpCAM/TROP1	IGF-II	LAP(TGF-b1)	Osteoprotegerin	TNF-beta
BMP-6	ErbB2	IL-1 alpha	Leptin R	PAI-I	TPO
BMP-7	ErbB3	IL-1 beta	LEPTIN(OB)	PARC	TRAIL R1
b-NGF	Erythropoietin R (EPO R)	IL-1 R4/ST2	LH	P-Cadherin	TRAIL R2
BTC	E-Selectin	IL-1 sRI	LIF	PDGF R alpha	TRAIL R3
CA125	Fas Ligand	IL-1 sRII	LIGHT	PDGF R beta	TRAIL R4
CA15-3	Fas/TNFRSF6	IL-10	LIMPII/SR-B2	PDGF-AA	Trappin-2/Elafin
CA19-9	Fcr RIIB/C	IL-10 R alpha	Lipocalin-2/NGAL	PDGF-AB	TREM-1
Carbonic Anhydrase IX(CA9)	Ferritin	IL-10 R beta	L-Selectin	PDGF-BB	TROY
Cardiotrophin-1 (CT-1)	FGF-4	IL-11	Lymphotactin	PECAM-1	TSH
Cathepsin S	FGF-6	IL-12 p40	LYVE-1	Platelet Factor 4	TSLP
CCL14a/HCC-1	FGF-7	IL-12 p70	Marapsin/Pancreasin	PIGF	u PAR
CCL21/6ckine	FGF-9	IL-13	MCP-1	Procalcitonin/Calcitonin	Ubiquitin+1
CCL28/VIC	FLRG	IL-13 Ra1	MCP-2	Prolactin	VCAM-1
CD14	Flt-3 Ligand	IL-13 Ra2	MCP-3	PSA-free	VE-Cadherin
CD23/Fc epsilon RII	Follistatin	IL-15	MCP-4	PSA-total	VEGF
CD27	Fractalkine	IL-16	MCSF	P-selectin	VEGF R2
CD30	FSH	IL-17	M-CSF R	RAGE	VEGF R3
CD40	Furin	IL-17B	MDC	RANK	VEGF-C
CD40 Ligand	Galectin-7	IL-17C	MICA	RANTES	VEGF-D
CEA	GCP-2	IL-17F	MICB	Resistin	
CEACAM-1	GCSF	IL-17R	MIF	S-100b	
CK beta 8-1	GDF-15/MIC-1	IL-18 BPa	MIG	SAA	

RayBiotech, Inc., the protein array pioneer company, strives to research and develop new products to meet demands of the biomedical community. RayBio's patent-pending technology allows detection of 274 cytokines, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable and cost effective. Products include: Cytokine Arrays, Chemokine Arrays, ELISA kits, Phosphotyrosine kits, EIA kits, Recombinant Proteins, Antibodies, and custom services.

1. Antibody arrays
2. Cytokine antibody array
    - Human cytokine antibody arrays
    - Mouse cytokine antibody arrays
    - Rat cytokine antibody arrays

   Pathway- or disease-focused antibody arrays
    - Inflammation antibody array
    - Angiogensis antibody array
    - Chemokine antibody array
    - Growth factor antibody array
    - MMP antibody array
    - Atherosclerosis antibody array
    - Adipokine antibody arrays

   Antibody analysis tool, software

3. ELISA
4. Cell-based phosphorylation assay
5. Custom antibody arrays
6. Antibody
7. Recombinant protein
8. Cytokine protein arrays
9. Quantibody arrays for quantitative measurement of cytokine and other protein concentration.
10. Phosphorylation antibody arrays
11. Biotin label-based antibody arrays for high density antibody arrays
12. EIA
13. Peptide

RayBiotech also provides excellent custom service:

1. Antibody arrays

2. Protein arrays
3. Peptide synthesis
4. Production of recombinant protein and antibody
5. Peptide arrays
6. Phosphorylation arrays
7. ELISA
8. EIA
9. Assay development

Just simply send your samples and we will do the assay for you.

Technology transfer program
Have you developed technologies or reagents of interest to the scientific and research community? RayBiotech can help you commercialize your technologies, reagents and dream.

VI. Troubleshooting guide

Problem	Cause	Recommendation
Weak signal or no signal	1. Taking too much time for Detection.	1. The whole Detection process must be completed in 30 min.
	2. Film developer does not work properly.	2. Fix film developer.
	3. Did not mix HRP-streptavidin well before use.	3. Mix tube containing 1,000X HRP-Conjugated Streptavidin well before use since precipitation may form during storage.
	4. Sample is too dilute.	4. Increase sample volume, (e.g. using undilute sample) or using more cells (e.g. seed 2 million cells. After 1 or 2 days, change complete medium with low serum medium and collect conditioned medium 2 day later. Use about 1 to 2 ml of conditioned medium for experiment).
	5. Other.	1. Reduce blocking concentration by diluting in 1X Wash Buffer II.
		2. Slightly increase HRP concentrations.
		3. Slightly increase biotin-antibody concentrations.
		4. Expose film for overnight to detect weak signal.
Uneven signal	1. Bubbles formed during incubation.	1. Remove bubble during incubation.
	2. Membranes were not completely covered by solution.	2. Completely cover membranes with solution.
High background	1. Exposure to x-ray file is too long.	1. Decrease exposure time.
	2. Membranes were allowed to dry out during experiment.	2. Completely cover membranes with solution during experiment.
	3. Sample is too concentrated.	3. Use more diluted sample.

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